Role of Integrin $\alpha_2\beta_1$ (VLA-2) in the Migration of Human Melanoma Cells on Laminin and Type IV Collagen

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The random cell migration of four human melanoma cell lines on laminin and type IV collagen–coated substrates was studied by video time-lapse image analysis and compared to the expression of a number of β_1 integrins including $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, and $\alpha_6\beta_1$ using flow cytometry. These integrins were heterogeneously expressed in the four cell lines tested with three of four lines expressing $\alpha_2\beta_1$. The melanoma cell line that did not express $\alpha_2\beta_1$ exhibited weak attachment and low cell migration rate on both laminin and type IV collagen, whereas the other melanoma cell lines showed an increase in attachment and mean cell migration rate in a dose-dependent manner on the matrix molecules ($p < 0.001$). The enhanced migration seen in the three cell lines could be specifically inhibited by function blocking anti-β_1 and anti-α_2 monoclonal antibodies ($p < 0.001$) but not by function blocking anti-α_3 and anti-α_6 monoclonal antibodies. Image analysis of the cells before and after treatment with anti-β_1 and anti-α_2 MoAb indicated that the inhibition of migration did not result in detectable cell detachment, retraction of cell processes, or other significant cell-shape change. Taken together, the findings suggest that the observable enhanced migration on laminin and type IV collagen of a number of human melanoma cell lines is largely mediated by integrin $\alpha_2\beta_1$.
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To invade, malignant tumor cells must be able to bind to the basement membrane, to digest it using proteinase activity, and to migrate into the surrounding tissue [1,2]. To metastasize, the invasive cells must penetrate the vascular structures, enter the circulation, and finally bind to and invade the host tissue at the site of secondary tumor formation. Two extra-cellular matrix (ECM) proteins, laminin and type IV collagen, are important components of the basement membrane at the dermal epidermal junction and in the vascular structures of the primary and secondary sites. Laminin and type IV collagen have been shown to promote the attachment and to stimulate the migration of many cell types *in vitro* [3–6] including melanoma [7–9] and the effects of these components on cells appear to be mediated by a number of cell surface adhesion receptors including the β_1 integrin family. The integrins are a set of protein heterodimers composed of one α and one β subunit. The β_1 integrin family, also known as the VLA (very late antigen), share a common β_1 subunit combined with one among at least six possible α subunits [10,11]. Among them, integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$ bind to laminin and type IV collagen; integrin $\alpha_3\beta_1$ binds to laminin, fibronectin, and type IV collagen; and $\alpha_6\beta_1$ is known as a monospecific laminin receptor. On the other hand, the heterodimers formed in combination with α_4 or α_5 subunits are known to be fibronectin receptors. Although non-integrin ECM receptors and recently characterized new α subunits have been identified, particularly in melanoma [12,13], β_1 integrin-mediated interactions with laminin and/or type IV collagen can be studied at least in part, in relation to the expression of $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$ and $\alpha_6\beta_1$ integrins.

The members of the β_1 integrin family have been intensively studied in relation to the malignant phenotype of diverse cell types [10]. Among the β_1-integrins binding laminin and type IV collagen, integrin $\alpha_2\beta_1$ may play a role in invasion and metastasis. This integrin has been recently reported to enhance the metastatic potential of rhabdomyosarcoma cells [14] and to be biochemically identical to a previously described "melanoma progression antigen" [15,16] and upregulated in highly aggressive melanoma cells [17].

To further define the role of certain β_1 integrins in human melanoma, we studied the expression of laminin and type IV collagen receptors of VLA-1 to VLA-6 on four melanoma cell lines including two primary and two metastatic melanoma cell lines. The functional significance of these laminin and type IV collagen receptors within the β_1 integrin family was studied by substrate matrix mole-

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Abbreviations:
BSA: bovine serum albumin
ECM: extra-cellular matrix
EDTA: ethylenediaminetetraacetic acid
LN: lymph node
MEM: minimal essential media
MFI: mean fluorescence intensity
MM-AN: metastatic melanoma cell line-code
MM-RU: metastatic melanoma cell line-code
MoAb: monoclonal antibody
PBS: phosphate-buffered saline
PM-WK: primary melanoma cell line-code
RGP: radial growth phase component
RPM-EP: recurrent primary melanoma cell line-code
VGP: vertical growth phase component
VLA: very late antigen

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cule mediated increase of cell attachment and migration rate and specific anti-integrin subunit monoclonal antibody mediated inhibition assays *in vitro*. Our data indicate that $\alpha_2\beta_1$ integrin plays an important role in laminin and type IV collagen mediated melanoma cell attachment and enhanced migration *in vitro*.

MATERIALS AND METHODS

Cell Lines and Cell Culture Human cutaneous melanoma cell lines were isolated as previously described [18]. Briefly, the PM-WK cell line was obtained from approximately 10% of the original sterile fresh surgical specimen of primary cutaneous malignant melanoma, superficial spreading type. The tissue fragments were washed and incubated in 0.25% trypsin at 37°C for 1 h. The epidermis was separated from the dermis using a dissecting microscope and a radial growth phase (RGP) culture was obtained from explants of epidermis in media non-supportive of keratinocytes. The RPM-EP cell line was established from a dermal nodule of a recurrent primary cutaneous melanoma, thus representing the vertical growth phase (VGP) of primary cutaneous melanoma. MM-AN and MM-RU cell lines were derived from lymph node metastases. All cell lines have exhibited stable morphologies and growth properties for over two years and were confirmed as melanoma cells by anti-S-100 protein antibody (Dako Corp) and anti-HMB-45 antibody (Enzo diagnostics, Inc.) testing. Precise characterization of these cell lines is described in our previous report [18]. All cells were cultured in minimal essential media (MEM) with Earles balanced salt solution supplemented with 2% fetal bovine serum and 8% newborn calf serum and penicillin, streptomycin, and amphotericin B (Gibco Catalog Number 600-5240AG Antibiotic-Antimycotic). The cultures were passed, at sub-confluence, approximately once a week, and kept in a 5% CO_2 and 95% air humidified atmosphere.

Antibodies and ECM Proteins Monoclonal antibody (MoAb) AIIB2 against the extra-cellular domain of the common β_1 integrin subunit of the VLA family [19] was kindly provided by Dr. Caroline H. Damsky (University of California, San Francisco, CA). The MoAb to $\alpha_1\beta_1$ (Ts2/7) was provided by Dr. Martin E. Hemler (Dana-Farber Cancer Institute, Boston, MA) [20]. The MoAbs to the α_2 subunit (P1E6) and α_3 subunit (P1B5) were purchased from Telios Pharmaceuticals, Inc. (San Diego, CA) [21]. The MoAb to the α_6 subunit (GoH3) was kindly provided by Dr. Arnoud Sonnenberg (Central Laboratory of the Netherlands, Amsterdam, the Netherlands) [22]. The MoAb P3 (anti-Fc receptor) [23] was utilized for flow-cytometry analysis as a negative control and MoAb W6/32 (specific for all major histocompatibility complex-I HLA heavy chains) was used as an unrelated specific MoAb control in the cell attachment and migration inhibition assays (American Type Culture Collection).

Laminin and type IV collagen (isolated by ion-exchange chromatography from the basement membrane of the Engelbreth-Holm-Swarm mouse tumor) were purchased from Collaborative Research Inc. (Bedford, MA).

Flow-Cytometry Analysis Melanoma cells were obtained in suspension after trypsin ethylenediaminetetraacetic acid (EDTA) or EDTA treatment alone of the sub-confluent cultures and incubated for 30 min at 4°C with the MoAbs (approximate immunoglobulin G concentration was 1 to 2 μg/ml) described above at dilutions of 1:10 (conditioned medium containing MoAb AIIB2) or 1:1000 (from ascites, all others), then washed three times and incubated for 30 min with a 1/40 fluorescein-labeled goat-anti-rat (for MoAb GoH3) or goat-anti-mouse (for the other MoAbs) antiserum (Cappel). The expression of β_1, α_1, α_2, α_3, and α_6 integrin subunits was analyzed using a Becton Dickinson FACScan II. Each cell line was also reacted with MoAb P3 (anti-Fc receptor) as a control.

Cell Attachment Assay Attachment assays were performed in 24-well plates (Primaria, Falcon, Lincoln Park, NJ) coated with the test ECM proteins followed by denatured bovine serum albumin (BSA) coating using established techniques. Briefly, laminin and type IV collagen were diluted to PBS to concentrations of 1.0, 10, and 100 μg/ml and 1 ml of each solution was added to each well. The plates were kept overnight at 4°C and washed three times with PBS, and residual protein binding sites were subsequently blocked by 12 h incubation with heat-denatured bovine serum albumin (Sigma, 20 mg/ml, 80°C, 20 min) at 4°C as previously described [5]. After three washings with PBS, 1 ml of serum-free medium (MEM) containing BSA (2 mg/ml) was added in each well and the plates were allowed to equilibrate at 37°C, 5% CO_2, 95% air for 30 min. Cells were obtained in single-cell suspension with 0.02% EDTA and washed twice, and, after trypan blue viability testing, 10^5 cells were added in each well and incubated at 37°C, 5% CO_2, 95% air for 60 to 90 min. Preliminary studies at 15-, 30-, 60-, 90-, and 120-min incubations determined that 15- and 30-min incubations were too short and 120-min too long to detect significant differences of cell attachment to the various substrates (data not shown). The wells were then uniformly washed three times with PBS to remove unattached cells and, lastly, the attached cells were collected by incubation with 0.25% trypsin and 0.02% EDTA (Gibco, Grand Island, NY) for 10 min, resuspended, and counted with a standard hemacytometer (Sigma). Each experiment was conducted in triplicate and mean attached cell number, standard deviation, standard error, and statistical significance (Student *t* test) were calculated.

Cell-Attachment-Inhibition Assay For inhibition experiments, the MoAbs were used at concentrations of 2 to 10 μg/ml using the following dilutions: 1:500 for W6/32 MoAb (used as a non-specific control MoAb), 1:3 for anti-β_1 AIIB2 (conditioned medium) MoAb, 1:500 for anti-α_2 P1E6 MoAb, and 1:500 for anti-α_3. These different MoAbs were added to the four different melanoma cell line suspensions prior to plating onto 96-well plates previously coated with either 0.1 ml of 10 μg/ml of collagen or laminin, followed by denatured BSA blocking as described above. Triplicate wells were seeded with 2.5×10^4 cells per well for each antibody/cell line mixture and following 60 min incubation, the number of attached cells was calculated by placing the center of the well under the 10× objective field of the image-analysis system (described below) and counting all the cells in the field. A cursor covered each cell to not count them twice. Counts in the field ranged from 35 to 680 cells. The ratio of the area of the entire well to the field area is 10 to 1, thus the total number of attached cells per well is 10 times the cell count. Each experiment was conducted in triplicate and the data analyzed as outlined in the section above.

Cell Migration Assay Micro-cover glasses (VWR Scientific Inc.) were coated with laminin, type IV collagen, and denatured BSA using the same procedure described for cell attachment assays and kept in 35-mm tissue culture dishes (Miles Laboratories). Then, 2.5 ml of serum free or 10% serum containing MEM was added and the dishes allowed to equilibrate at 37°C, 5% CO_2, 95% air for 30 min and approximately 10^5 cells were seeded on micro coverslips to obtain a cell density between 0.4 and 1.0 cells/10^4 mm^2 as previously described [18]. Briefly, cell cultures were observed under a 10× phase-contrast objective with a Nikon Diaphot inverted microscope partially encased in a Plexiglas incubator housing and attached Nikon incubator NP-2 with stage thermostat and an air/CO_2 flow mixer to obtain constant temperature and pH. Cell movements were recorded with a video camera attached to the video camera port and connected to a time-lapse video cassette recorder. Cell migration was recorded for 4 h between 4 to 30 h after plating. Previous experiments have shown that no significant difference exists between mean migration rates obtained from observations 4 h and 30 h after plating nor significant differences between serum or serum-free media if performed during the same interval (data not shown). Each migration experiment recorded the pathways of 25 to 50 cells, and each experiment was repeated three times at each coating concentration of ECM component for calculation of mean migration rate standard deviation, standard error, and statistical significance (Student t test).

To determine the mean migration rate, the video images were played back at 1-h intervals and images were digitally saved and migration pathways measured using a Microcomp image-analysis

system and software (Southern Micro Instruments, Atlanta, GA), using a personal computer with a video card using frame-grabbing digitizing software and a high-resolution video monitor. The data were saved as an MS-DOS (Microsoft) file and translated through a network (TOPS, Sun Microsystem Co., Berkeley, CA) to a Macintosh SE for statistical analysis. Normalized migration paths were obtained by "grabbing" individual cell paths and "dragging" them without rotation so that the origin of all paths were superimposed on one central point. Phase-contrast photomicrographs were taken with a Nikon FE-2 camera attached to the inverted microscope.

Cell-Migration–Inhibition Assay To detect changes in cell migration by the addition of function-blocking MoAb, the lowest substrate concentration were tested that demonstrated significant enhanced migration in each cell line (RPM-EP, MM-AN, and MM-RU on 10, 100, and 10 μg/ml coating of laminin, respectively, and 10 μg/ml of collagen for all three lines). Because the line PM-WK did not show significant enhancement by either substrate, it was arbitrarily tested at 10 μg/ml to control for non-specific or non-integrin mediated effects of the various MoAbs tested. The medium surrounding previously attached cells (for at least 4 h) was exchanged with temperature and pH-equilibrated MEM containing the various function-blocking and unrelated control MoAbs at the same concentrations described in the attachment-inhibition assays. The preparations were further equilibrated for 30 min before recording the migration pathways. Cell area and cell shape (cell form factor) was quantified by image analysis prior to addition of MoAb and at the end of the assay to determine if reduction in migration was accompanied by cell rounding (retraction of processes) and/or detachment. Mean cell area and mean cell form factor was determined on 10 randomly chosen cells (the first 10 cells encountered from the top of the video field) in each initial and final time frame for each experimental condition (MoAb and substrate). Area and form factor were calculated by planar morphometry software of the image-analysis system described in the previous section. Cell form factor is defined as $4\pi \times (\text{area})/(\text{perimeter})^2$ and is equal to 1 for a circle and increasingly less than 1 with increasing irregularity or angularity (elliptical, polygonal, polygonal with scalloping, dendricity, tripolar, bipolar) [24]. Statistical analysis of migration rates was performed as described above and initial and final cell area and cell form factors were compared by paired Student t test.

RESULTS

Differential Expression of β_1 Integrin Subunits (VLA-1 to VLA-6) to Laminin and/or Type IV Collagen in Human Melanoma Cell lines There was heterogeneous expression of β_1 integrin subunits; each of our melanoma cell lines exhibited a unique complement despite identical culture conditions (Fig 1). All lines expressed the β_1 integrin subunit as detected by the MoAb specific for this subunit: mean fluorescence intensity (MFI) was 27.0 in PM-WK, 120.0 in RPM-EP, 150 in MM-AN, and 79.0 in MM-RU.

Despite the heterogeneous expression among all the laminin and collagen binding integrin subunits tested, the most frequent expression was the α_2 subunit, with expression in three of the four lines tested. The α_2 integrin subunit appeared on the lines derived from later stages of tumor progression: expression was moderately detected ($10 \leq \text{MFI} < 50$) in the recurrent and metastatic cell lines RPM-EP, MM-AN, and MM-RU (MFI = 38.3, 38.1 and 17.3 respectively). In contrast, the RGP primary melanoma derived cell line, PM-WK, showed a very low expression of the α_2 integrin subunit (MFI = 4.5) (Fig 1*A*).

The other integrin subunits tested were less frequently represented. There was absent or very low expression of the α_1 subunit on PM-WK and MM-RU (MFI < 5) and low expression of the α_3 subunit on PM-WK (MFI < 10) whereas RPM-EP and MM-AN expressed moderate levels of the α_1 and α_3 subunits ($10 \leq \text{MFI} < 50$) (Fig 1*B*). Moderate expression of the α_6 subunit was present only in MM-AN (MFI = 13.8) whereas the others showed absent or very low expression (MFI < 5). Table I summarized the findings.

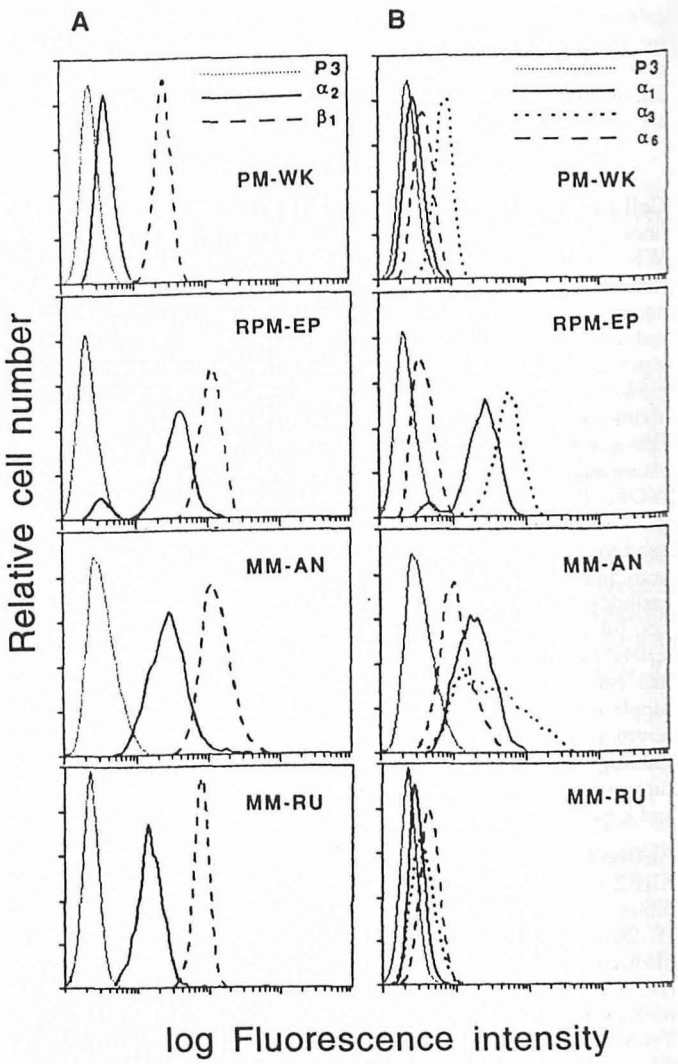

Figure 1. Integrin subunit expression in human melanoma cells analyzed by flow cytometry. *A*) The level of β_1 integrin subunit expression *(dashed line)* in the melanoma cell line PM-WK *(top)* is less than the cell line RPM-EP *(second from top)* or the two metastatic melanoma cell lines (MM-AN, *third from top*) and (MM-RU, *bottom*). The α_2 (VLA-2) expression *(solid line)* on PM-WK *(top)*, is low or nearly absent, whereas there is moderate expression on RPM-EP cells and the metastatic cell lines. Negative control *(fine dotted line)* following incubation with anti-Fc receptor MoAb (P3). *B*) The α_1 *(solid line)*, α_3 *(coarse dotted line)*, and α_6 *(dashed line)* (VLA-1, 3, and 6, respectively) subunit expression on PM-WK, RPM-EP, MM-AN, and MM-RU.

The pattern of expression of these integrins on each cell line has remained constant over the last 2 years.

Differential Cell Attachment of Melanoma Cell Lines to Laminin or Type IV Collagen–Coated Substrates We investigated the attachment properties of these four human melanoma cell lines to substrates with increasing coating concentrations of laminin and type IV collagen. RPM-EP, MM-AN, and MM-RU showed significantly enhanced attachment to laminin ($p < 0.001$) and to type IV collagen ($p < 0.001$) in a dose-dependent manner (see Fig 2). In contrast, PM-WK exhibited only a slight enhancement of attachment only to laminin and no enhancement to type IV collagen even at high coating concentrations.

To further evaluate the role of β_1 integrins in cell attachment of these melanoma cell lines to laminin and type IV collagen, we conducted attachment inhibition experiments with these melanoma cell lines using function-blocking MoAbs directed against the

Table I. Summary of Flow-Cytometry Data[a] of Expression of Integrin Subunits Tested

Integrin Subunit	Cell Line: Primary Melanoma Origin PM-WK RGP Derived	RPM-EP VGP Derived	Metastatic Melanoma Origin MM-AN LN Derived	MM-RU LN Derived
β_1	++	+++	+++	+++
α_1	−	++	++	−
α_2	−	++	++	++
α_3	+	++	++	−
α_6	−	−	++	−

[a] MFI was converted into semi-quantitative format as follows: MFI < 5.0 defined as very low or absent (−), 5.0 ≤ MFI < 10 as low (+), 10 ≤ MFI < 50 as moderate (++), and MFI ≥ 50 defined as high (+++). RGP, radial growth phase component; VGP, vertical growth phase component; LN, lymph node.

β_1, α_2, α_3, or α_6 subunits that have been reported to have an inhibitory effect on cell attachment [19,21,25,26]. Incubation with an unrelated control MoAb, anti-α_3, and anti-α_6 MoAb, did not significantly inhibit cell attachment of all four cell lines to collagen or laminin (see Fig 3). Furthermore, function-blocking anti-α_2 and anti-β_1 MoAbs had no effect on attachment of the line PM-WK. However, anti-β_1 MoAb significantly inhibited ($p < 0.001$) the attachment of the other three melanoma cell lines to type IV collagen and two of the three lines to laminin (see Fig 3). The cell line RPM-EP showed a slight but not significant reduction in binding to laminin with this MoAb.

Likewise, treatment with function-blocking anti-α_2 also significantly reduced attachment ($p < 0.001$) of the MM-RU cells to both collagen and laminin (Fig 3*d*). In contrast, the anti-α_2 significantly reduced attachment of the MM-AN cells to type IV collagen but not laminin and this MoAb did not significantly reduce attachment of RPM-EP to laminin or type IV collagen (Fig 3*b*,*c*).

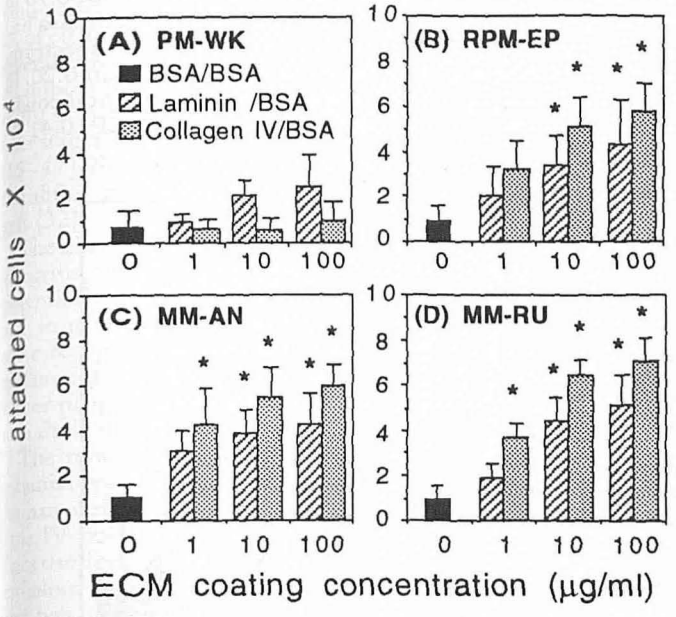

Figure 2. Melanoma cell attachment assay to heat-denatured BSA/BSA coated substrates or to various concentrations of laminin/BSA or type IV collagen/BSA coated substrates. The melanoma cell line PM-WK (*A*) exhibits less attachment to both laminin and type IV collagen than the other cell lines (RPM-EP, MM-AN, MM-RU; *B*, *C*, and *D*, respectively). The latter three lines (*B*, *C*, and *D*) show a significant dose-dependent increase in cell attachment on both laminin and type IV collagen (*$p < 0.001$). *Error bars*, standard deviation.

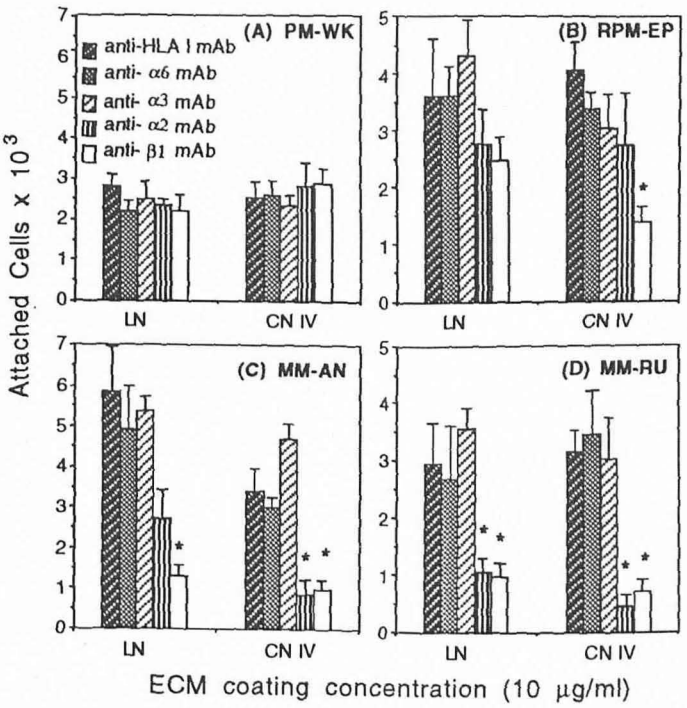

Figure 3. Cell adhesion assay on the effect of incubation with function-blocking MoAbs directed against the β_1, α_2, α_3, or α_6 subunits as compared to an unrelated control MoAb (anti-HLA class I). Incubation with the anti-α_3 and anti-α_6 MoAb did not inhibit cell attachment of all four cell lines to type IV collagen or laminin. The anti-α_2 (AIIB2) or anti-β_1 (P1E6) MoAb showed variable ability to inhibit PM-WK cells (*A*), RPM-EP cells (*B*), MM-AN cells (*C*), or MM-RU (*D*) cells to both laminin and collagen. (*$p < 0.001$). *Error bars*, standard deviation.

Differential Cell Migration of Melanoma Cell Lines on Laminin and Type IV Collagen The effect of laminin and type IV collagen-coated substrates on the mean random migration of human melanoma cells was investigated by image analysis of time-lapse video recordings of individual cell migration pathways as described in *Materials and Methods*. The migration pathways revealed marked heterogeneous cell behavior among the cell lines in response to increased substrate-coating concentrations of matrix molecules. Intrinsic differences in mean migration rates among the lines were identified; however, comparisons within a particular cell line to control substrates (BSA) and different ECM substrate concentrations was most helpful to evaluate the differential haptokinetic responses.

The mean random migration rates of all four cell lines in serum-free media on control heat-denatured BSA coated substrate were 2, 4, 4, and 15 µm/h for PM-WK, RPM-EP, MM-AN, and MM-RU, respectively. Indeed, the four cell lines maintained approximate relative intrinsic migration rates; PM-WK showed the lowest migration rate, RPM-EP and MM-AN intermediate rates, and MM-RU the highest migration rate. In media containing 10% serum or in serum-free media (to eliminate a possible contribution of plasma fibronectin that could potentially coat the substrates during the experiment), a marked increase in mean migration rates on increasing coating concentrations of laminin and collagen was observed. No significant difference in migration rate on the coated substrates was observed between serum or serum-free media.

The mean migration rates for all cells in each experiment using the different ECM components was calculated and can be seen plotted as vertical bars in Fig 4. The mean random migration rate of three of the four cell lines was significantly enhanced ($p < 0.001$) by both laminin and type IV collagen-coated substrates in a dose-dependent manner (Fig 4). However, the primary melanoma cell line PM-WK showed only a mild but not significant increase in

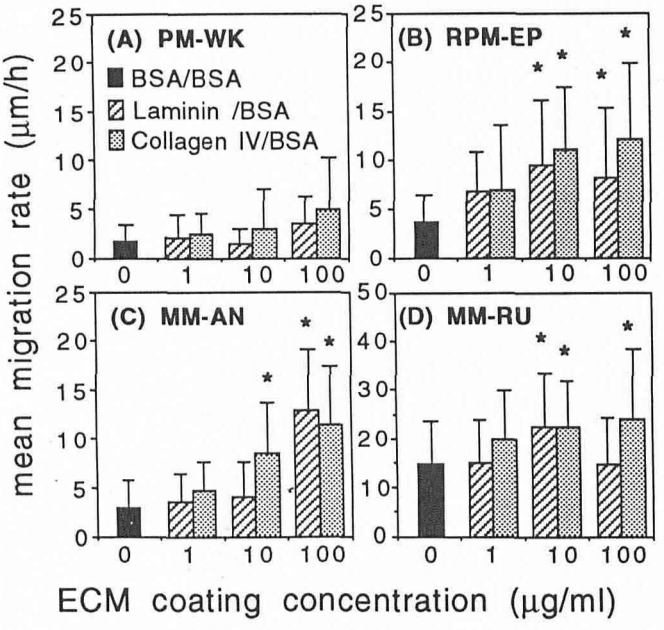

Figure 4. Bar graph of the mean random migration rates of PM-WK (*A*), RPM-EP (*B*), MM-AN (*C*), and MM-RU (*D*) on laminin- or type IV collagen–coated substrates shows a dose-dependent and significant enhancement of migration in the latter three cell lines. (*$p < 0.001$). *Vertical bar,* mean migration rate derived from the migration paths of 75 to 150 cells. *Error bars,* standard deviation.

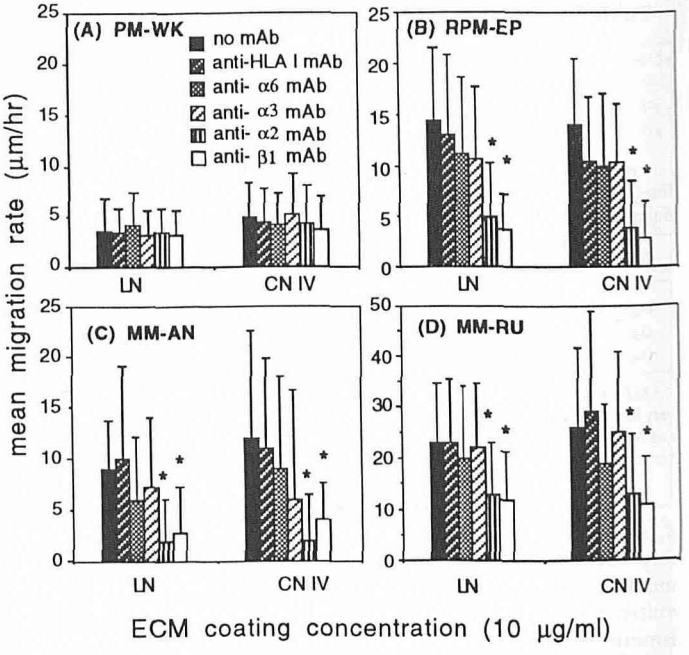

Figure 5. Cell-migration assay on the effect of incubation with function-blocking MoAbs directed against the β_1, α_2, α_3, or α_6 subunits as compared to no MoAb treatment or to an unrelated control MoAb (anti-HLA class I). The anti-β_1 MoAb (AIIB2) and anti-α_2 MoAb (P1E6) did not inhibit the migration of the PM-WK cell line (*A*), whereas they significantly inhibit the migration rate of the RPM-EP (*B*), MM-AN (*C*), and MM-RU (*D*) cell lines on laminin- or type IV collagen–coated substrates (*$p < 0.001$). *Vertical bars,* migration paths of approximately 25 to 50 cells. *Error bars,* standard deviation.

migration rate on these substrates. Interestingly, the enhanced migration rate of MM-RU cells on laminin at 10 μg/ml coating concentration was not observed at the highest coating concentration (100 μg/ml). A similar pattern on type IV collagen was observed with RPM-EP, although to a lesser extent because it was still significantly higher than the control migration rate on BSA alone. To investigate a role for β_1 integrins known to bind laminin or collagen in mediating the enhanced haptokinesis, we performed a number of function-blocking MoAb inhibition assays, described below.

MoAb Inhibition of Enhanced Melanoma Cell Migration with Function-Blocking Anti-β_1 and Anti-α_2 To explore possible inhibition of the migration of the four melanoma cell lines on laminin and type IV collagen–coated substrates, we used the function-blocking MoAbs directed against the β_1, α_2, α_3, or α_6 subunits that were employed in the cell-attachment studies outlined above. These MoAb have been reported to have an inhibitory effect on cell attachment to these matrix molecules [19,25,26].

Incubation with the unrelated control MoAb (W6/32), the function-blocking anti-α_3, or anti-α_6 MoAbs did not significantly inhibit cell migration of all four cell lines on collagen or laminin coated substrates (see Fig 5). Likewise, function-blocking anti-α_2 and anti-β_1 MoAb had no effect on cell migration of the line PM-WK (Fig 5*a*). However, both anti-β_1 and anti-α_2 MoAb had a statistically significant inhibitory effect ($p < 0.001$) on the migration rate of RPM-EP, MM-AN, and MM-RU (Fig 5*b,c,d*). Figure 6 shows the effect of variable anti-β_1 and anti-α_2 MoAb concentration on the migration of MM-RU cells. The anti-α_3 and anti-α_6 MoAbs were also tested at three- to tenfold higher concentrations and no significant inhibition was detected in all the cell lines.

The time-lapse video tapes revealed that treatment of the melanoma cells with the MoAbs, in particular the anti-β_1 MoAb or anti-α_2 MoAb, did not induce detachment of the cells from either the laminin- or collagen-coated substrates, even following several hours of incubation, because the cells continued to migrate at rates of 3–5 μm/h for RPM-EP and MM-AN and 10–12 μm/h in the case of MM-RU. Image analysis of individual cells using planar morphometry before and after addition of MoAb provided further evidence that the inhibition of migration by the MoAb was not the result of cell detachment or cell retraction of processes. The cell form factor (see *Materials and Methods*) for round, detached cells resting on the substrate before cell spreading ranged from 0.76 to 0.93 with a mean of 0.87 ± 0.02. Well-spread round cells also had form factors above 0.75 whereas, at the other end of the spectrum, bipolar or dendritic cells have form-factor values less than 0.20. In fact, the mean cell form factors in the various experimental conditions ranged from 0.46 ± 0.3 to 0.62 ± 0.4 for RPM-EP, 0.41 ±

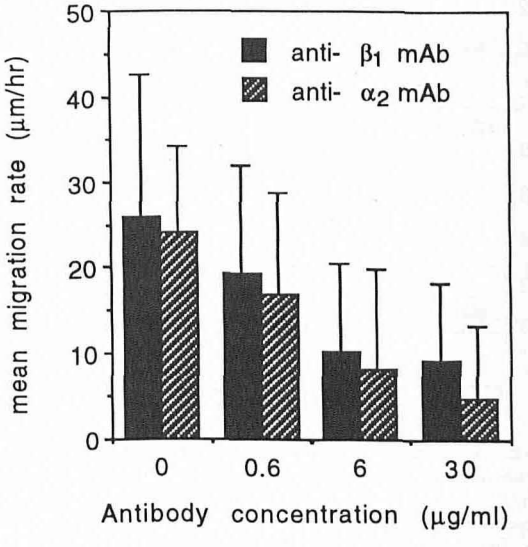

Figure 6. Effect of increasing concentration of the anti-β_1 MoAb (AIIB2) and anti-α_2 MoAb (P1E6) on the migration rate of MM-RU cells. *Error bars,* standard deviation.

0.03 to 0.56 ± 0.05 for MM-AN, and 0.43 ± 0.04 to 0.52 ± 0.05 for MM-RU and no significant difference was detected using paired Student t test between initial and post-MoAb treatment using anti-β_1 and anti-α_2 MoAb on either laminin or collagen substrates. Likewise, mean cell areas (±SD) on the matrix molecule–coated substrates before and after MoAb treatment ranged from 968 ± 79 to 1249 ± 122 μm^2 for RPM-EP, 520 ± 48 to 707 ± 99 μm^2 for MM-AN, and 701 ± 85 to 990 ± 85 μm^2 for MM-RU. No significant difference in projected area was detected using paired Student t test between initial and post-MoAb treatment using anti-β_1 and anti-α_2 MoAb on either laminin or collagen substrates. As a reference, mean projected area of non-attached, round cells resting on the substrate immediately after cell plating and before cell spreading ranged from 97 to 215 μm^2. Taken together, the decreased migration but not total inhibition of migration and the area and form-factor data indicate that the inhibition of migration by the anti-β_1 and anti-α_2 MoAb did not involve cell retraction of processes, detachment, and/or cell rounding.

DISCUSSION

Cell adhesion and cell migration are essential steps in the invasive and metastatic behavior of malignant cells. Malignant cell binding to the basement membrane and cell migration are believed to be involved in primary-site infiltration during the invasive step of tumor progression and in the colonization of the secondary site in the metastatic step [1]. Some of the members of the β_1 integrin family are specifically directed towards components of the basement membrane. Among them, the $\alpha_1\beta_1$, $\alpha_2\beta_1$, and $\alpha_6\beta_1$ integrins are well characterized and exhibit a high affinity for laminin and/or type IV collagen, which are two major components of the basement membranes [11,13,27]. New β_1 integrins continue to be characterized, including $\alpha_7\beta_1$, a laminin receptor that has been described in melanoma cells [12,13]. We have restricted our study to VLA-1 to VLA-6 integrins because of the availability of these antibodies for detection and, more importantly, function-blocking studies. The β_1 integrins appear to be connected by their intra-cellular domain to components of the cytoskeleton [28,29] and may participate in the mechanisms responsible for active cell movement. Moreover, laminin and type IV collagen have been shown to enhance the motility of either normal or malignant cell types *in vitro* [3–9,30,31] and several lines of evidence suggest that the $\alpha_2\beta_1$ integrin appears to be an important marker for the malignant phenotype [14] and especially in melanoma where it has been shown to be associated with later stages of tumor progression and aggressive melanoma cell lines [15–17]. Furthermore, anti-β_1 and anti-α_2 MoAbs have been shown to inhibit migration of tumor cells in three-dimensional collagen gels [32].

The aim of this study was to further investigate the role of the β_1 integrins that bind to laminin and type IV collagen in two of the potentially critical steps of invasion and metastasis: cell adhesion and, in particular, cell motility. We investigated the expression of these receptors in a number of our recently characterized human melanoma cell lines that were isolated from different stages of tumor progression to detect differences that might provide insight into their different behavior on specific ECM components.

The relative expression of the β_1, α_1, α_2, α_3, and α_6 integrin subunits was elucidated by flow cytometry on four of our cultured human melanoma cell lines. The expression of these laminin and/or type IV collagen receptors is heterogeneous among the four cell lines studied, similar to previous studies in melanoma [16,33]. Nevertheless, heterogeneous expression determines differential adhesive behavior to specific ECM molecules [34] or relates to tumor progression [16,17]. Although our sample of cell lines studied is small because our primary focus is on cell-migration functional assays, it is interesting nevertheless that the α_2 subunit was common to the three cell lines derived from later stages of tumor progression, consistent with the findings by Klein *et al* [16]. Furthermore, the lack of expression of this integrin in PM-WK cells derived from a pre-invasive melanoma and the expression in invasive and metastatic derived cells also supports the work describing it as a marker of melanoma progression [15,16]. Future migration functional studies directed towards the β_3 integrin subunit, another receptor showing expression in melanoma from later stages of progression [33], may lead insight into other integrins involved in cell motility.

The attachment of human melanoma cells derived from invasive or metastatic tumors was significantly enhanced by substratum-bound laminin or type IV collagen in a dose-dependent manner. The intra-epidermal derived cell line (PM-WK) did not show enhanced attachment to collagen in the coating concentrations tested, and although mild enhanced attachment to laminin was observed, it was not as significant as the invasive or metastatic lines derived cell lines. This observation may relate to the low invasiveness and lack of metastatic behavior displayed by intra-epidermal primary melanoma cells pathologically [35–37] and experimentally [38,39].

To characterize which integrins were the predominant ones involved in cell attachment to type IV collagen and laminin in these human melanoma cell lines, we incubated the cells with a number of function-blocking MoAbs including anti-β_1, anti-α_2, anti-α_3, and anti-α_6 MoAbs. We found that the attachment profiles of all melanoma lines in the presence of these MoAb to be heterogeneous, reflecting, at least in part, the heterogeneous expression of β_1 integrins on these cell lines as determined by flow cytometry. The PM-WK cell line, expressed little or no α_2, α_3, or α_6, attached poorly to both substrates and the function-blocking MoAb against these integrins had no effect on binding. Surprisingly, anti-β_1 did not completely inhibit this mild attachment, even though the cells express β_1 integrin, suggesting that other non-β_1 integrin collagen and laminin receptors are mediating this low level of attachment. In contrast, the cell line MM-RU, expressing only the α_2 subunit among the VLAs tested, was highly adhesive to type IV collagen and laminin and this attachment was significantly inhibited by anti-β_1 MoAb as well as with anti-α_2 MoAb, suggesting that most of the β_1 integrin related adhesion to these ECM substrates is mediated by $\alpha_2\beta_1$ integrin.

The other two cell lines showed intermediate profiles of attachment in the inhibition assays. The anti-β_1 antibody inhibited attachment of the MM-AN cells (which expresses the β_1, α_2, α_3, and α_6 subunits) to both collagen and laminin and the anti-α_2 MoAb inhibited attachment to type IV collagen and reduced but did not significantly inhibit the attachment to laminin, indicating that the α_3 or α_6 subunits may contribute to binding to this latter substrate. However, the MoAb against the α_3 or α_6 subunits did not inhibit this attachment, suggesting that the presence of another β_1 integrin subunit that is involved in binding to laminin is sufficient to permit adhesion. Again, it is possible that it is the $\alpha_7\beta_1$ laminin receptor recently reported on melanoma cells [12,13]. Likewise, the RPM-EP cell line (which expresses the β_1, α_2, and α_3 subunits) also had a unique attachment profile with the MoAb treatment: the anti-β_1 MoAb was able to inhibit significantly attachment to type IV collagen but not laminin and the anti-α_2 did not inhibit significantly attachment to both substrates. These findings lend further support that certain melanoma cell lines utilize non-β_1 integrins for cell attachment. Other β integrin subunits may be involved, including β_3 integrin subunit as indicated above [33].

We were not able to detect inhibition of attachment of any of the cell lines with the anti-α_3 or anti-α_6 MoAbs alone. Future studies using combinations of these and other function-blocking MoAbs may help dissect the relative contribution of adhesion receptors for each cell line; however, numerous studies have pursued this approach and, because we detected a significant contribution of $\alpha_2\beta_1$ in cell attachment, we wished to focus on the possible role of $\alpha_2\beta_1$ on cell migration.

The effect of laminin- and type IV collagen–coated substrates on melanoma mean random cell migration (haptokinesis) *in vitro* was similarly examined by stimulation and inhibition assays on the four melanoma cell lines. A significant dose-dependent acceleration of the migration rate on laminin and type IV collagen was observed in

the $\alpha_2\beta_1$-positive cell lines. In contrast, the PM-WK cell line remained significantly less motile on both substrates and lacked $\alpha_2\beta_1$ integrin. The lack of $\alpha_2\beta_1$ expression, poor attachment to the substrate, and low migration rate may be related to other factors affecting motility such as down-regulation of certain cytoskeletal proteins [40]. Nevertheless, the relationship of $\alpha_2\beta_1$ integrin expression to the acceleration in cell migration induced by laminin- and type IV collagen-coated substrates was indicated by the experiments demonstrating the significant inhibition of acceleration by the addition of either anti-α_2 or anti-β_1 MoAb. Surprisingly, unlike the attachment studies, the RPM-EP, MM-AN, and MM-RU cell lines all showed similar migration-inhibition profiles following treatment with the various function-blocking MoAbs. The lack of concordance between ability of anti-α_2 or anti-β_1 antibodies to inhibit attachment and to inhibit migration on laminin or collagen in the RPM-EP and MM-AN cell lines may be due to reports that specific integrins involved in initial cell adhesion can differ from those involved in migration [32]. It is possible that production of endogenous matrix molecules in the longer migration assay may contribute to this finding; however, the discrepancy observed in the RPM-EP and MM-AN lines may also be the result of the other integrin subunits that were identified that are known to promote adhesion but apparently do not play as critical a role in cell migration as the α_2 subunit. Cell locomotion involves not only adhesion, but also signal transduction to engage the cytoskeleton associated proteins, as well as other molecules for subsequent cyclical attachment and detachment. Nevertheless, the similar inhibition of migration to baseline (denatured BSA rate) by only anti-α_2 or anti-β_1 MoAb suggests that most of the enhanced migration is due to $\alpha_2\beta_1$. The reduction in migration is not due to detachment or rounding of the cells due to retraction of cell processes because the morphometric data indicate no significant change in projected surface area or shape following treatment with anti-α_2 or anti-β_1. Presumably the other integrin and non-integrin receptors prevent retraction of the cell processes. The mechanism by which this integrin enhances cell migration on collagen and laminin is unclear, but may be simply due to increased traction afforded by the direct binding to ligand or may involve a complex pathway involving signal transduction to engage cytoskeletal proteins. Recent studies on integrin-mediated enhanced migration on fibronectin suggests that cell tension transmitted to the ECM via the cytoskeleton may involve the association of β_1 integrins with specific actin-associated proteins including talin, vinculin, and/or α-actinin [28,41-43]. Furthermore, migration of fibroblasts on type I collagen has been shown to be inhibited by the same MoAbs we have used in this study, and regulation of this $\alpha_2\beta_1$ mediated migration is mediated in part by shifts in concentrations of extracellular Mg^{++} and Ca^{++} [44].

Taken together our data indicate that cultured melanoma cells demonstrate heterogeneous behavior not only with regard to cell attachment but also cell migration on laminin and/or type IV collagen. These data also confirm that these differences are largely mediated by differential expression of members of the β_1 integrin family. That the $\alpha_{2\beta_1}$ heterodimer may play a functionally predominant role is suggested by the following data: 1) the low motility on both substrates and the low expression of $\alpha_2\beta_1$ in the PM-WK cell line; 2) $\alpha_2\beta_1$ is the only β_1 integrin expressed in all three cell lines that demonstrate high affinity and increased migratory response to both substrates; 3) the greatest motility in these four cell lines was observed in the line expressing only $\alpha_2\beta_1$; and 4) the anti-α_2 subunit MoAb reproduced the inhibitory effect of anti-β_1 MoAb on cell migration rate on both substrates in all three lines.

Our data are in accordance with the previously described effect of laminin and type IV collagen on melanoma cell migration [3-9] and indicate a role of at least some β_1 heterodimers, and, in particular, $\alpha_2\beta_1$, for binding to these substrates. The present data support the hypothesis of the importance of the expression of $\alpha_2\beta_1$ as a malignant phenotype [14-17] and indicate a functionally predominant role for this integrin not only in melanoma cell attachment but also cell migration on laminin and type IV collagen *in vitro*. These findings may provide further insight into understanding melanoma metastases *in vivo*.

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